Utility of molecular analysis of peritoneal fluid in staging laparoscopy of advanced oesophagogastric junction and gastric cancer prior to neoadjuvant treatment

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Abstract

The significance of molecular analysis of peritoneal fluid in staging laparoscopy of oesophagogastric junction (EGJ) and gastric cancer is still controversial.

METHODS: A retrospective analysis of prospective data collection of all patients diagnosed with locally advanced gastric neoplasia or Siewert II-III EGJ cancer from July 2009 to October 2019 who underwent staging peritoneal lavage prior to neoadjuvant treatment was performed. Cytology studies and molecular analysis of peritoneal lavage were performed by real-time reverse transcriptase polymerase chain reaction (RT_PCR) of carcinoembryonic antigen (CEA) and cytokeratin 20 (CK20) as tumor makers.

RESULTS: 168 patients were operated. Citology and molecular analysis were performed in 138 patients. Macroscopic carcinomatosis (P+) was evidenced in 12.3% (17 patients). Of the remaining 87.7% (121 patients), 9.9% (12 patients) had positive cytology. In 21% (29 patients) disseminated macroscopic or microscopic disease (P + or Cyt+) was identified. 11.6% (16 patients) were P0Cyt- but RT-PCR+. Of these, 9 responded to chemotherapy before salvage surgery.

The sensitivity of cytology and molecular analysis was 0.70 and 0.76, respectively (p = 0.67). The survival of Cyt-RT_PCR + vs. Cyt + RT_PCR + patients was similar (p = 0.1). However, there were no differences in the survival curves between Cyt-RT_PCR- patients vs Cyt-RT_PCR + patients who underwent salvage surgery (p = 0.69).

CONCLUSION: Molecular analysis showed a sensitivity of 76%. In our study, the survival of P0 Cyt-RT_PCR + patients responding to chemotherapy and undergoing surgery was similar to that of P0 Cyt-RT_PCR- patients but less disease free survival.

Introduction

Gastric carcinoma is the fifth most frequent neoplasm worldwide [1], representing the third leading cause of death from cancer. Despite the advances in neoadjuvant therapies, the prognosis of this disease is still poor, mainly due to late diagnosis in Western countries.

The clinical guidelines of most international societies such as the National Comprehensive Cancer Network (NCCN) [2], European Society for Medical Oncology (ESMO) [3], European Society of Surgical Oncology (ESSO) [4], or Japanese Gastric Cancer Association (JGCA) [5] among others, include staging laparoscopy +/- peritoneal lavage as a fundamental tool for correct tumour evaluation, especially in advanced stages (Ib-III). The objective is to rule out macroscopic and/or microscopic peritoneal dissemination to avoid unnecessary neoadjuvant treatments. However, there is still controversy about the best technique to detect the presence of free intraperitoneal tumor cells (Cyt+) or the most adequate therapeutic management of patients without macroscopic peritoneal disease (P0) but who are Cyt+ (P0 Cyt+), especially in those in whom the cytology becomes negative after chemotherapy [6-7].

Regarding the method to analyse the presence of cells in the peritoneal fluid, most authors use citology (Papanicolaou stain) despite having a highly variable sensitivity of between 11-67% [7-10]. There are other techniques such as molecular analysis of peritoneal fluid using reverse transcriptase-polymerase chain reaction (RT_PCR) of proteins including carcinoembryonic antigen (CEA) or cytokeratin 20 (CK20), which have shown a higher sensitivity in some series [9-12] (up to 77% [13-14]). However, the prognostic value of this technique is controversial [9,12] especially in patients who are Cyt- but RT_PCR+, and molecular analysis was not included in their therapeutic algorithms.

The objective of this study was to analyse the sensitivity of molecular analysis by RT_PCR of CEA and CK20 in peritoneal lavage during staging laparoscopy in our centre in advanced gastric or Siewert II-III EGJ neoplasia. The secondary objective was to compare the survival of Cyt-RT_PCR+ vs. Cyt-RT_PCR- to elucidate the prognostic role of this technique and its utility in the therapeutic algorithm of these patients.

Material And Methods

Since 2005, all patients diagnosed with gastric neoplasia or Siewert II-III oesophagogastric junction (EGJ) cancer are studied in the Functional Unit of Oesophagogastric Tumors (UTEG) in our centre. For extension study, thoracoabdominal computed tomography (CT) is performed with the addition of endoscopic ultrasound (EUS) when CT results require a change in therapeutic management. Since July 2009, laparoscopic staging is systematically added (and laparoscopic staging with peritoneal lavage and citology since January 2010), when all the following criteria were met:

1.- Locally advanced gastric or Siewert II-III EGJ adenocarcinoma (≥ cT3 or cN+ according to the TNM [15]) staged by thoracoabdominal CT +/- EUS

2.- Absence of metastasis in the preoperative extension study (cM0).

3.- Patient is a candidate for neoadjuvant treatment.

4.- Patients with no previous surgical history that contraindicate the performance of a laparoscopic staging approach and who provide informed consent to undergo surgery.

Since April 2011, RT_PCR analysis of CEA and CK 20 in peritoneal fluid and Papanicolaou staining are routinely performed in all lavage-cytology of gastric neoplasms or Siewert II-III EGJ. Figure 1 shows the diagnostic-therapeutic algorithm of our centre.

Study design

We performed a retrospective analysis of a prospective data collection of all patients diagnosed with gastric neoplasia or locally advanced Siewert II-III EGJ from July 2009 to October 2019 who underwent laparoscopic staging with peritoneal lavage and cytology prior to neoadjuvant treatment.

Age, sex, comorbidity, surgical risk according to the *American Society of Anesthesiologists* (ASA) scale [16], cTNM classification [15], and neoadjuvant treatment post-laparoscopy were evaluated. Pathological variables registered during staging laparoscopy were: presence of macroscopic implants (P+), presence of neoplastic cells in the peritoneal fluid using the Papanicolaou stain or immunocitochemical expression of (Cyt+), and presence of neoplastic cells in the peritoneal fluid according to RT_PCR of CEA and CK 20 (RT_PCR +).

Postoperative morbidity was registered according to the Clavien-Dindo classification [17]. Postoperative mortality was established in-hospital or 30-day.

Staging laparoscopy. Technique

The patient is placed in the supine position and pneumoperitoneum is performed. Three trocars are inserted: 1 of 11 mm at the umbilical level and 2 of 5 mm on both flanks. Ascites fluid, if present, is collected for cytological and molecular analysis using a Nelaton 14 Fch probe (Pennine, UK), and the abdominal cavity is meticulously examined for peritoneal implants. The entire parietal peritoneum is examined from both diaphragms, flanks to the pelvis and pouch of Douglas. Examination continues with the round ligament, greater and lesser omentum, the serosa of the intestinal loops and the angle of Treitz. Subsequently, the liver surface is examined in search of subcapsular infracentimetric metastases not detected by CT. Tumour resectability is assessed by elevating the left liver lobe and exposing the stomach to rule out infiltration of neighbouring structures. In doubtful cases, the minor sac is opened and infiltration of the posterior gastric face to retroperitoneal structures is evaluated. It is important to emphasise that large dissections and manipulations of the tumour should be avoided, since the objective of laparoscopy is to rule out macroscopic implants and wash-cytology collection without damaging other structures and with minimal tumour manipulation.

Finally, 1 L of 0.9% physiological saline is introduced by means of an aspirator-irrigator (Coloplast, HU), the abdominal cavity is shaken and 50 mL of each of the following 3 spaces is collected by means of a Nelaton probe: left subphrenic, subhepatic and fundus of sack of Douglas. Half of the liquid is sent for cytological study and the other half for molecular study.

Cytological study

The material from each lavage is centrifuged separately. If a suitable pellet is obtained, a cell block is made and two extensions are fixed in 96° alcohol and subsequently stained with Papanicolaou stain. If a suitable pellet is not obtained, 3 cytocentrifuges are performed, fixed with 96° alcohol, with one being stained with Papanicolaou and the other two being reserved in alcohol in case an immunocytochemical study is necessary.

In the case of poorly cohesive gastric adenocarcinoma (World Health Organisation [WHO] classification) / diffuse (Laurens classification), an immunocytochemical study is always performed. CEA and CK20 expression is determined in the cell block or in the reserved centrifuges. If the adenocarcinoma is tubular

(WHO classification) / intestinal (Laurens classification), no immunohistochemical study is performed and the reserved cytospins are stained with Papanicolaou.

Each lavage of the different locations is diagnosed independently.

A sample is considered positive for malignant cells when atypical epithelial cells or CEA positive and/or CK20 positive cells are observed. Otherwise the case is considered negative.

Molecular analysis

RNA isolation

The lavage samples were centrifuged at 1500 rpm for 15 min at 4 °C. The supernatant was removed from each sample and the remaining pellets were suspended in 500 µL of TRIzol reagent (Invitrogen) and processed with the, Direct-zol™ RNA MiniPrep spin column purification kit according to the manufacturer's instructions (Zymo Research).

Real-time RT_PCR

RNA concentrations were determined spectrophotometrically. First strand complementary DNA (cDNA) was synthesised from total RNA (1 mg), using the SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies). The cDNA samples were stored at -20 °C.

TaqMan Assays-on-Demand Gene Expression Assay primers for CE, CK20, and b2-microglobulin (BMG) mRNA were purchased from Applied Biosystems.

Real-time quantitative RT_PCR was performed using the Rotor-Gene 5 Plex (Corbett). DNA was amplified in a 20mL reaction containing cDNA, TaqMan Gene Expression Assay and Taqman Master Mix.

Each PCR reaction was subjected to 10 min at 95 °C, followed by 45 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds. Each run was assayed with positive and negative PCR controls. The endogenous control gene, BMG, was used to confirm the presence of mRNA in the peritoneal lavage samples. The optimal cut off for cycle amplification (CEA and CK20) was chosen using a receiver operating characteristic (ROC) curve determined in a previous study.

Samples above the cutoff value for CEA or CK20 mRNA were considered positive.

STATISTICAL ANALYSIS

Quantitative variables were expressed as mean +/- standard deviation or median +/- range. The categorical results were expressed as percentages.

To analyse the morbidity and mortality of staging laparoscopy, 168 patients who underwent staging laparoscopy were analysed. However, the analysis of sensitivity and survival only included patients who

had undergone both techniques (cytology plus molecular analysis) (n=138 patients). Macroscopic findings during laparoscopy were used as the gold standard.

ROC curves were used to compare sensitivities.

Survival analysis was performed using the Kaplan-Meier method, and the Log Rank test was used to compare survival. The statistical analyses were carried out with the statistical package SPSS version 18 (IBM, Armonk, New York, USA). Statistical significance was considered with $p < 0.05$ in all cases.

All data generated or analysed during this study are included in this published article.

Results

Of the 168 patients studied, 65% were men with a mean age of 60.8 +/- 11.1 years; 12.5% had underlying lung disease and 7.1% had heart disease as a notable comorbidity. More than half of the patients had an ASA 1-2 surgical risk (61.9%). Regarding clinical staging, the majority were cT4 (80.4%) and cT3 (9.5%), with cN + lymph node involvement in 94.6%.

A minimally invasive approach was used for lavage in 95.3% of the cases (160 patients). The conversion index was 3% (5 cases), the most frequent cause being intra-abdominal bleeding. Of these, only 2 patients required a packed red blood cell transfusion. The mean surgical time was 40.9 +/- 18.3 minutes. Postoperative morbidity was present in 3% (5 patients), being of minor complications (Clavien 2) in all of them. The postoperative mortality was 0%. The mean hospital stay was 1.06 +/- 2.7 days with a median of 0 days (0-21).

Cytological analysis of peritoneal fluid

Of the 138 interventions performed with cytology and molecular analysis, disseminated macroscopic or microscopic disease (P+ or Cyt+) was identified in 21% of the cases (29 patients), changing the staging to M1.

P+ was evidenced in 12.3% of the cases (17 patients). 9.9% of P0 patients (12 cases) were Cyt+, the molecular analysis being positive in all of them. In 14.7% of P0Cyt- patients (16 cases) the molecular analysis was positive. In total, 23.1% of the cases (28 patients) had microscopic disease not visualised during laparoscopy. The results are shown in greater detail in Figure 2.

In the 16 P0Cyt-RT_PCR+ patients chemotherapy was indicated. Of these, 9 patients (56.2%) presented frank radiological response, for which radical resective surgery was indicated. In the remaining 7 patients (43.8%) surgery was not indicated due to: tumour progression during chemotherapy treatment in 5 cases, appearance of a second neoplasm in a polypathological patient in 1 case, and lost to follow-up in 1 patient.

Comparison of cytology vs molecular sensitivity

Of the 138 peritoneal lavages performed, the cytological analysis was positive in 17.3% (24 patients) and molecular analysis was positive in 29.7% (41 patients) (Figure 2).

The sensitivity of cytology was 70% vs 76% of RT_PCR analysis, without statistically significant differences (p = 0.67) (Figure 3).

Survival comparison

The 2-year survival of M1 patients (P+ or Cyt+) compared to M0 patients (P0 or Cyt-) was 21.7% versus 71.1%, respectively, being statistically significant (p = 0.000).

When comparing Cyt+ and Cyt- patients at 2 years, the survival was 12.5% versus 65%, respectively (p = 0.000) (Figure 4A). The presence of macroscopic versus no macroscopic implants (P+ Cyt+ vs. P-Cyt+) did not influence survival (p = 0.63) (Figure 4B).

Regarding the molecular analysis, RT_PCR+ patients presented a worse survival than RT_PCR- patients (p = 0.000) (Figure 5A). No statistically significant differences were observed on comparing Cyt-RT_PCR+ vs. Cyt+RT_PCR+ patients, (p = 0.1) (Figure 5B). There were no differences in the survival curves on comparing Cyt-RT_PCR+ patients who responded to chemotherapy and underwent rescue surgery (9 patients) with Cyt- RT_PCR- patients (p = 0.69) (Figure 6A). However, disease-free survival is less in the first group (p=0.005) (Figura 6B).

Discussion

In this study, molecular analysis of CEA and CK 20 by RT_PCR presented a sensitivity similar to that of conventional cytology.

There is great controversy in the literature regarding the sensitivity and prognostic value of molecular analysis during staging laparoscopy in advanced gastric adenocarcinoma, and international clinical guidelines are far from providing an adequate answer to that question. This debate is especially focused on P0 Cyt- RT_PCR+ patients (11.6% in our study, lower than others like 19% published by Wong [9]).

Many of the studies available have shown that the sensitivity of molecular analysis is superior to that of cytology [9,14,28,19-22]. Moreover, in our study we did not find this. Considering that the sensitivity of our molecular analysis (76%) was similar to other groups published [9], the absence of statistically significant differences between the two techniques could be due to the high sensitivity of our cytology tests (70%), which was higher than others, like 61% reported by Wong [9]. Immunocitochemical study has surely helped to get this high sensitivity of citology test.

The specificity of molecular analysis varies greatly among the publications available, being superior to cytology such as in the meta-analysis by Xiao [22] but not in other studies such as that by Fujiwara *et al* [21]. The latter author highlighted a non-negligible number of false positives due to the presence of non-tumour cells, such as macrophages or leukocytes, secreting CEA in the peritoneal fluid.

Regarding the prognostic value of molecular analysis, based on the TNM classification, a positive molecular analysis cannot be considered M1 [15], and thus, an RT_PCR+ result is not comparable to Cyt+. However, most authors seem to agree that RT_PCR+ patients have a worse prognosis compared to RT_PCR- patients, with a higher recurrence rate and a worse long-term survival [14,21-22], and defend that molecular analysis may contribute to the identification of patients at high risk of peritoneal recurrence. For example, in the meta-analysis by Pecqueux *et al*. [18], the prognostic value of cytological and molecular analysis was similar, although the authors stated that prospective trials are necessary before validating their recommendation in clinical guidelines. In the meta-analysis by Deng *et al*. [12] the subgroup of RT_PCR+ patients showed a higher risk of mortality, recurrence, and peritoneal recurrence. Our results were similar, showing a worse survival for RT_PCR+ compared to RT_PCR- patients (p = 0.000). Furthermore, on comparing Cyt-RT_PCR+ and Cyt+RT_PCR+ patients, there were no differences in terms of survival; that is, the presence of a positive or negative cytology did not affect survival if the patients had a positive molecular analysis result. Moreover, if we consider that molecular analysis is a feasible and reproducible technique in referral centers, and its cost are not expensive, that could be reasonable to add it routinely during peritoneal lavage analysis.

However, on analysing the subgroup of Cyt-RT_PCR+ patients who responded to chemotherapy treatment and who underwent resective surgery, the survival was similar to that of the Cyt-RT_PCR- group (p = 0.69). This result supports performing surgery in these patients despite presenting a positive molecular analysis, similar to what has been described in other studies. However, disease-free survival of the first group is worse (p=0.005). In this subgroup of patients some authors had suggested more aggressive perioperative treatment to avoid intraperitoneal disease, such as intraperitoneal chemotherapy associated with surgical resection plus hyperthermic intraperitoneal chemotherapy [14,18,22,23].

The contributions of this study are the high sensitivity of the cytological analysis (superior to that of the majority of published series), which is of great importance when diagnosing metastatic patients, probably due to immunocitochemical study, the possibility of performing laparascopy under an outpatient surgery regime and the identification of the subgroup of patients P0Cyt-RT_PCR+ who would most benefit from surgical treatment, being those who present frank radiological response after chemotherapy.

The most relevant limitation of this study is the modest sample size. Although at the state level there are no previously published references, we only have 16 patients in the subgroup P0Cyt-RT_PCR+, and only 9 of them were operated. The statistical power of the study might be insufficient with respect to the analysis of this subgroup.

In conclusion, in our study molecular analysis of CEA and CK 20 by RT_PCR presented a sensitivity similar to that of conventional cytology, but identifies over 11.6% of subgroup of patients with a worse prognosis (P0 Cyt-RT_PCR+) . As molecular analysis is a feasible and reproducible technique in referral centers, we recommend the combining of molecular analysis and cytology in peritoneal lavage of

advanced gastric and Siewert II-III EGJ neoplasms. It would be necessary randomized trials to analyze the best treatment in the subgroup of patients P0Cyt-RT_PCR+.

Conclusions

Molecular analysis of peritoneal fluid after laparoscopy-lavage has a sensitivity of 76%, similar to citology. In our study, P0 Cyt-RT_PCR + patients who responded to chemotherapy and underwent surgery showed a survival similar to that of P0 Cyt-RT_PCR- patients, but less disease free survival. Randomized trials are necessary to determine the therapeutic implication of molecular analysis.

Declarations

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AUTHOR'S CONTRIBUTION: Miró M: protocol, data collection, data analysis, manuscript writing. Vives R : data collection, manuscript writing. Farran L: protocol, final revision. Secanella L: protocol, data analysis. Varela M and Baixeras N: cytology and molecular analysis, manuscript writing. Estremiana F, Bettonica C and Aranda H: protocol, data collection, final revision. Galán M: protocol, final revision. All authors had approved the submitted version

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ETHICS APPROVAL: Written informed consent was considered not necessary for the study, as it was a retrospective analysis of our usual everyday work. Data of patients were anonymized for the purposes of this analysis. The confidential information of the patients was protected according European normative. This manuscript has been revised for its publication by the Research Ethics Committee of Bellvitge University Hospital.

CONFLICTS OF INTEREST: The authors have no conflicts of interest to declare

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Figures

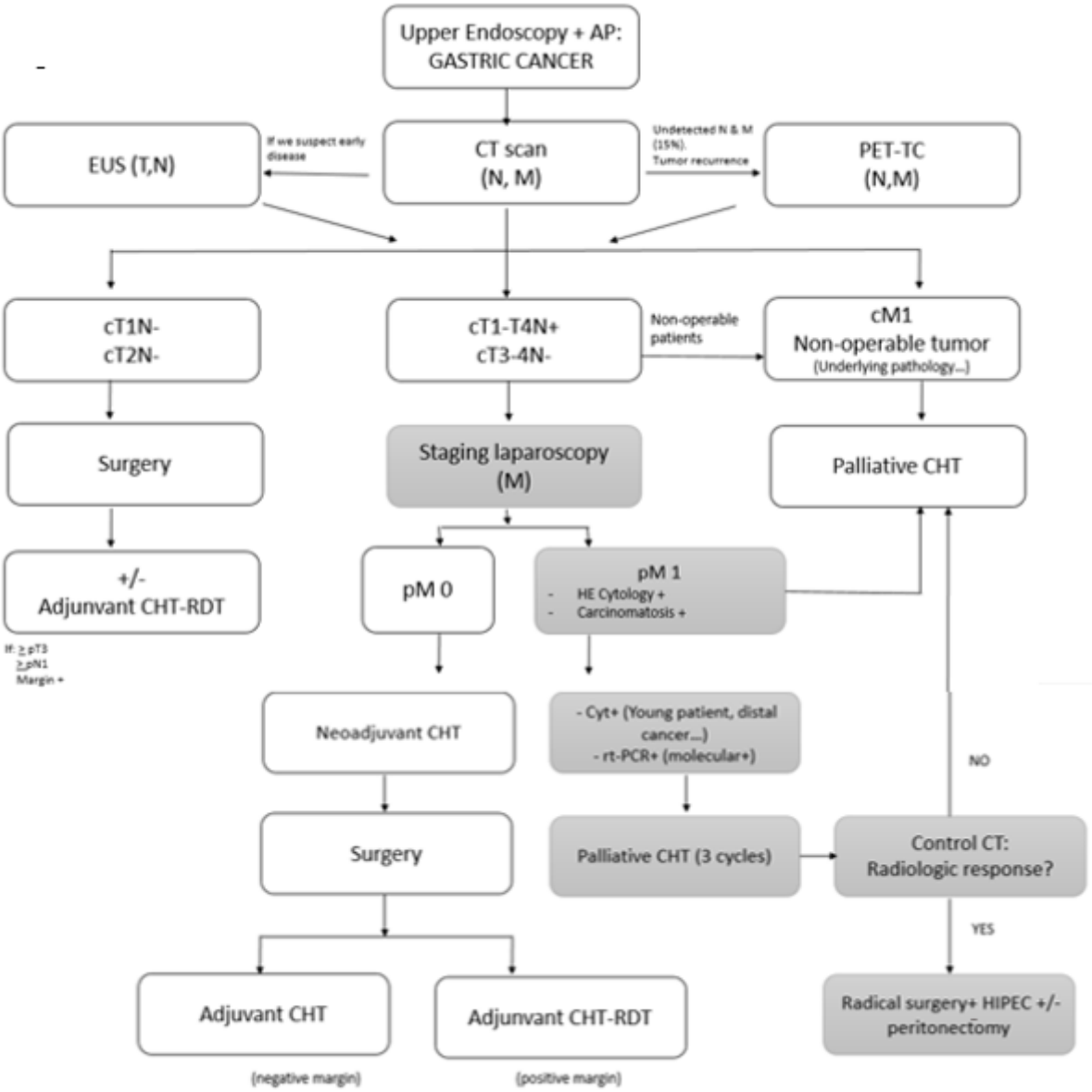

Figure 1

Diagnostic-therapeutic algorithm

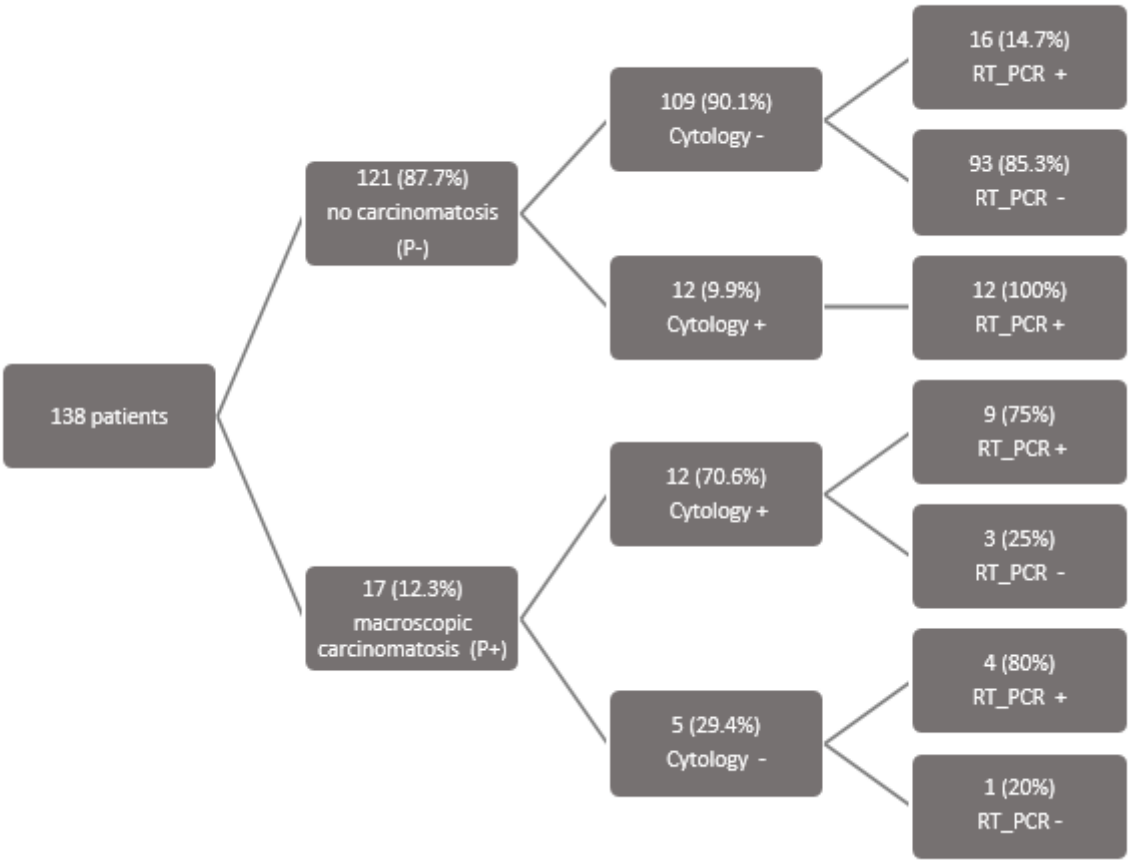

Figure 2

Results of patients undergoing cytology and molecular analysis (n=138)

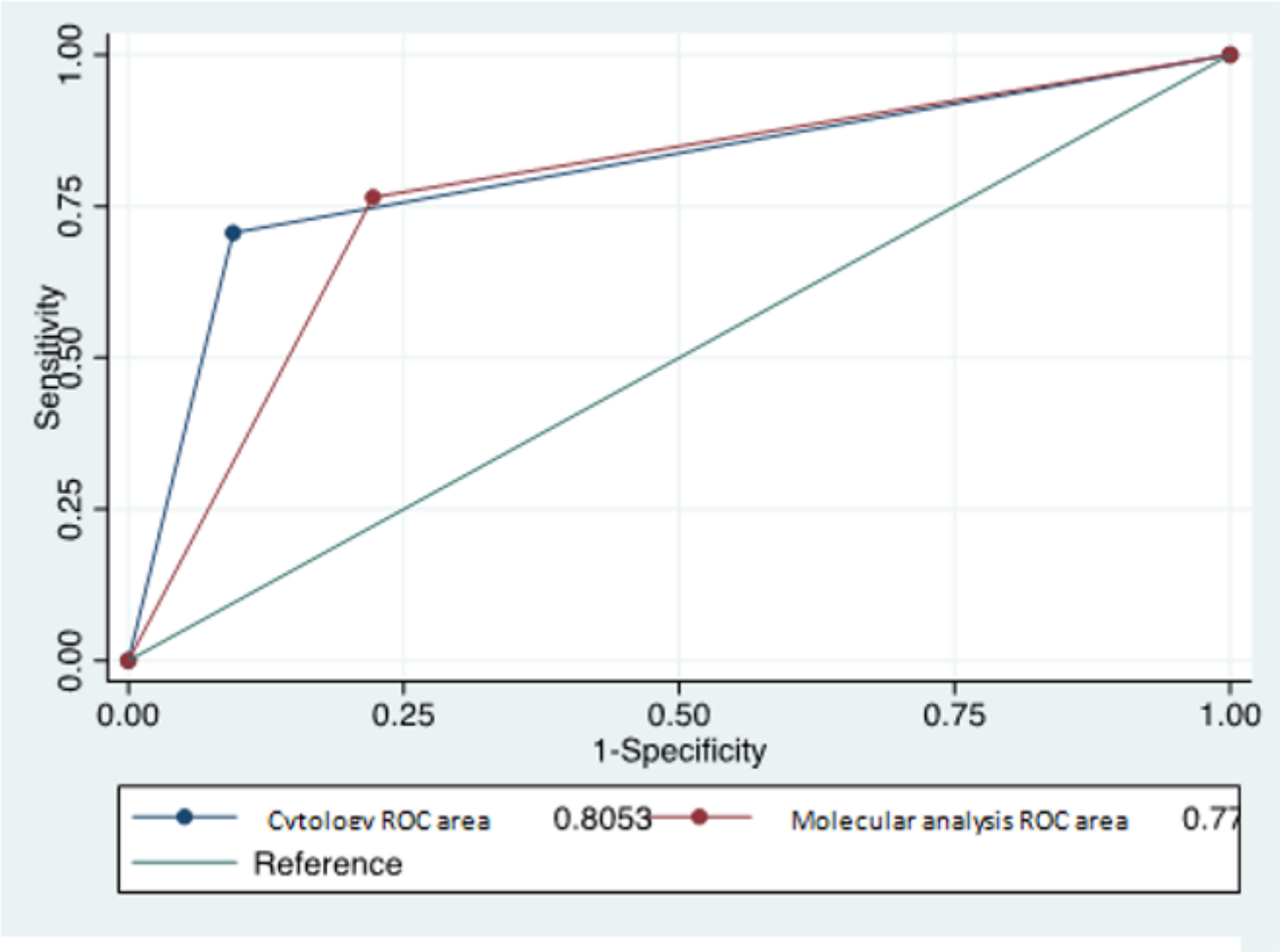

Figure 3

The receiver operating characteristic (ROC) curves for cytology and molecular detection of peritoneal dissemination (p = 0.64)

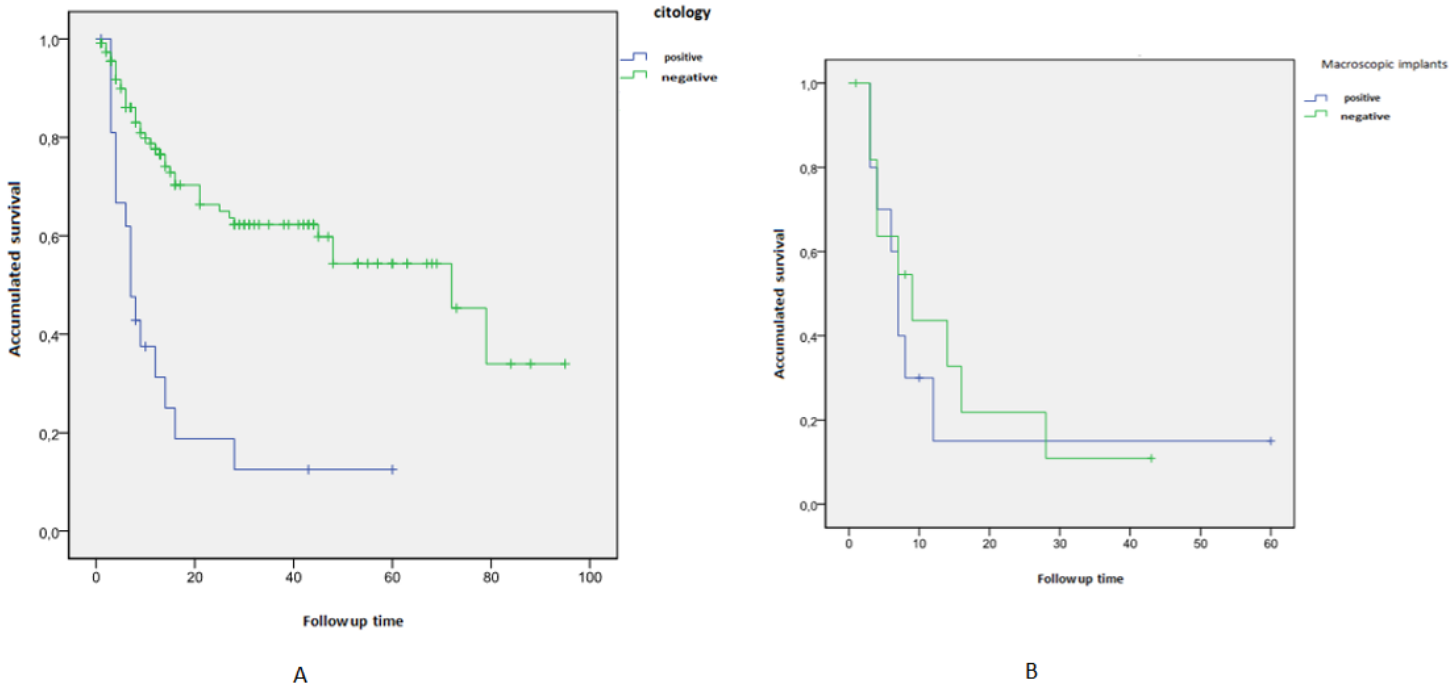

Figure 4

A. Survival curve of Cyt+ vs. Cyt- patients (p = 0.000). B. Survival curve of P-Cyt+ vs. P+Cyt+ patients (p = 0.63)

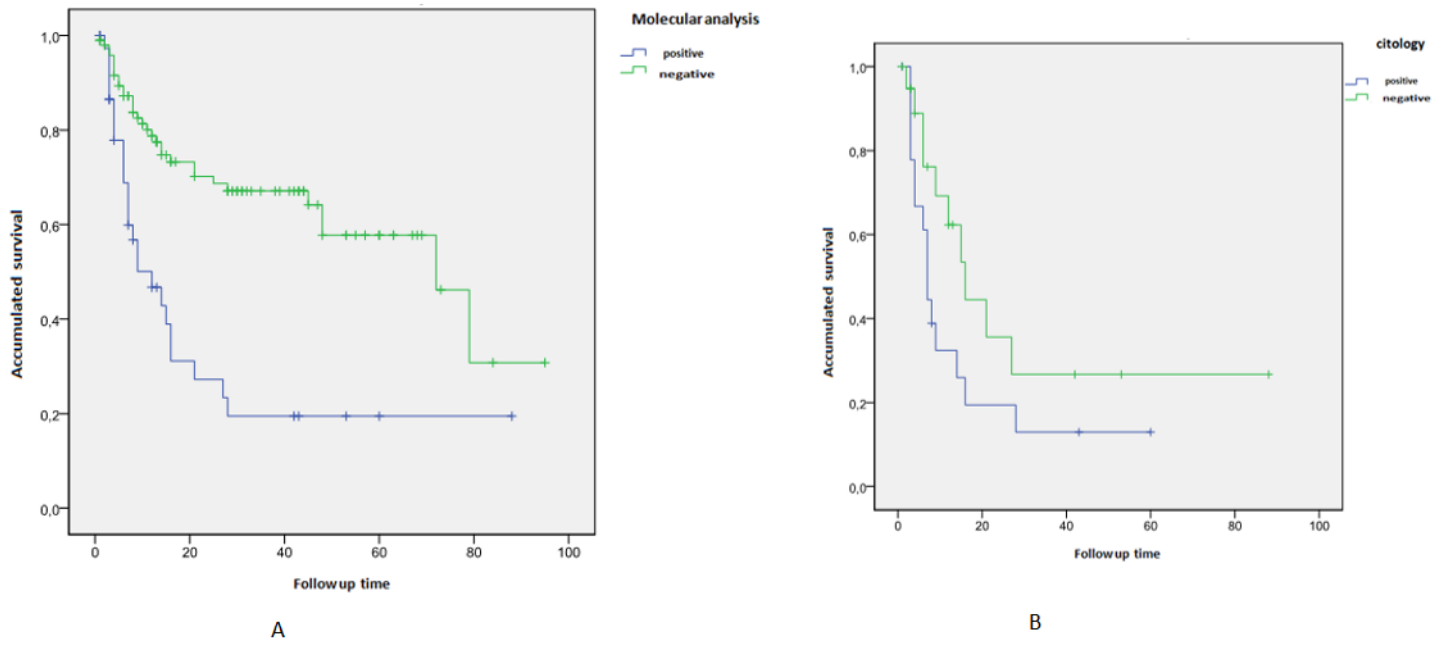

Figure 5

A. Survival curve of RT-PCR+ vs. RT-PCR- patients (p = 0.000). B. Survival curve of Cyt+RT-PCR+ vs. Cyt-RT-PCR+ patients (p = 0.121).

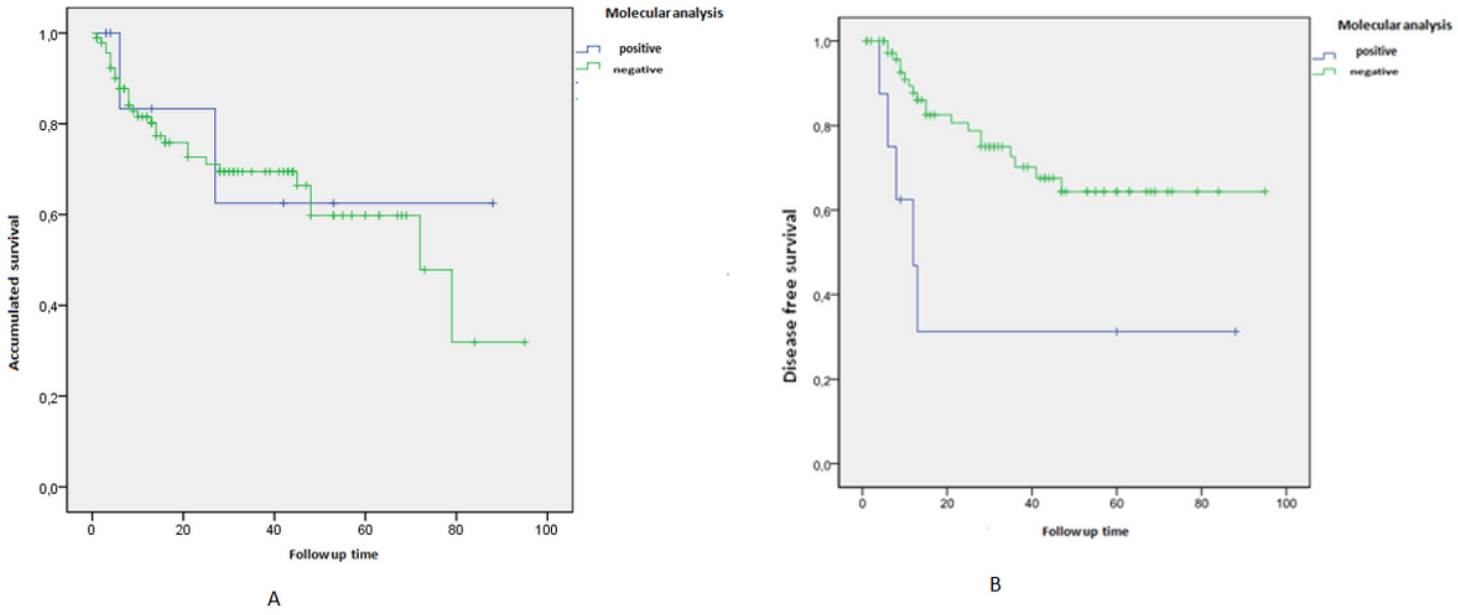

Figure 6

A. Survival curve of Cyt- rt-PCR- vs. Cyt-rt-PCR+ patients who underwent surgery after responded to chemotherapy treatment (p = 0.686). B. Disease-free survival curve of Cyt- rt-PCR- vs. Cyt-rt-PCR+ patients who underwent surgery after responded to chemotherapy treatment (p=0.005)